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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): ALVING et al

Serial No.: 09/859,389 Examiner: Cheu

Filed: May 18, 2000 Art Unit 1641

Title: DETECTION OF ANTIBODIES TO SQUALENE IN SERUM

DECLARATION UNDER 37 C.F.R. §1.132

1. I, Dr. Carl R. Alving, am a joint inventor of the invention subject to this patent application and make this declaration based upon personal knowledge in support of the patentability thereof.

2. I have reviewed and analyzed the primary reference relied upon by the Examiner in rejecting the claims of the application Asa et al US 6,214,566 (hereafter "the Asa Patent").

3. The technical merit and hypothesis suggested by Asa in the patent and collateral papers has been vigorously criticized and rejected by the scientific community as evidenced by my own published "Letters to the Editor" appearing in *Experimental and Molecular Pathology*, 68 196-198 (2000) (a true reprint copy attached hereto under Tab 1) and the subsequent report by the *Institute of Medicine* (a true copy attached hereto under Tab 2). Those documents detail the flawed scientific techniques used and conclusions reached by Asa.

4. While the Asa patent reports the existence of an assay for antibodies to squalene, it is non-specific to other antigenic epitopes (See Col. 7 lines 1-6). The Asa

patent is not enabling of the invention disclosed and claimed in our patent application. Asa does not teach or suggest the identification of an antibody specific to squalene and/or an antibody exhibiting strong dose dependant binding to squalene. That is, Asa does not teach or enable a method for detecting antibodies to squalene that have little or no cross reactivity to squalane as admitted in Asa's patent at Column 7 and also does not enable or identify monoclonal antibodies specific to squalene.

5. Your Declarant says nothing further.

VERIFICATION

I hereby declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Date: 2/2/04

Dr. Carl R. Alving

Experimental and Molecular Pathology 68, 196–198 (2000)
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LETTERS TO THE EDITOR

To the Editor:

A recent article in this journal by Asa *et al.* (2000) purports to measure serum antibodies to squalene. The paper fails to establish the validity of the test. The essential flaws involve selection of proper positive controls and proper negative controls, quantitative methods, and selection of study populations.

The authors hypothesize that antibodies are induced by "the adjuvancy of squalene," such that injection of squalene could elicit antibodies to squalene. One approach might be to inject squalene into an experimental animal to determine *first* whether the injection can induce the purported antibodies and *second* whether the assay can detect the induced antibodies. Antibodies induced by injection, if they exist, could then serve as a positive control for the unvalidated assay.

The assay describes no positive controls that actually validate the assertion of detecting antibodies to squalene. Such positive controls would consist of comparable serum samples demonstrated to contain anti-squalene antibodies after injection with squalene.

The authors assert that they have positive controls, in the form of two human subjects previously injected with a squalene-containing placebo during a clinical trial at the National Institutes of Health. However, the authors provide no preinjection results to establish that intentional injection of squalene led to antibodies to a substance already present in the body.

The assay also lacks elementary negative controls routinely run in enzyme-linked immunoassays. Such negative controls are required to prove that the assay is not detecting cross-reacting substances. In a new, unproven assay that claims to detect a novel antibody, one must prove specificity. There were no negative controls in which the human serum containing the presumed antibodies was omitted or in which the avidin-conjugated horseradish peroxidase was omitted. There is no evidence that the assay was not simply measuring other IgG molecules with nonspecific binding to squalene. This could be easily accomplished by substituting an oil molecule similar to squalene. An excellent negative control would be squalane, the fully hydrogenated form of squalene.

The unknown human serum samples were tested only at a single dilution (1:400). Most assays for naturally occurring antibodies, particularly antibodies to lipids, start at a higher concentration of serum, typically a dilution of 1:50. Thus, the method of Asa *et al.* could miss the presence of antibodies detectable at a higher concentration of serum. It is possible that normal blood donors could give positive results at a higher concentration of serum.

A further drawback of using only a single dilution of serum, rather than a series of dilutions, is that there is no way to obtain a quantitative measure of the degree of activity in the sample. Titers are routinely obtained when antibody levels are measured. The absence of quantitation in this assay weakens meaningful comparisons between unknown serum samples from subjects accrued in a nonrandom manner.

Figure 1, said to show "antisqualene antibody responses," is particularly flawed. In this figure, unspecified quantities of squalene were added as aqueous dilutions of 1:10, 1:100, 1:1000 and 1:10,000 for impregnation of nitrocellulose. No explanation is provided for how an oil such as squalene, not soluble in water, could be diluted in water by the published methods. Further, a washing solution containing polyoxyethylene sorbitan monolaurate could have detergent-like qualities that could remove squalene. Despite the extensive dilutions of the squalene, there is no evidence of a dilution curve (assessing each strip vertically), regardless of whether the antibody reactions were rated as 3+, 2+, or 1+. This suggests that nonspecific binding of serum immunoglobulin may have occurred.

The conclusions of Asa and colleagues, purporting to correlate anti-squalene with Gulf War illnesses, in our opinion, rely on circular logic. Positive results with an assay not previously validated to detect antibodies cannot be used as scientific proof that antibodies to the antigen exist in samples of unknowns. It is premature to proceed directly to testing

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TAB 1

serum samples from healthy people and sick people before conducting the fundamental validation steps.

The critique offered here is not meant to imply that antibodies to squalene do not or cannot exist. As pointed out by the authors, extensive work demonstrates that antibodies to cholesterol, a molecule for which squalene serves as a precursor, are found in virtually all normal human sera. A recent report proposes that naturally occurring antibodies to cholesterol may serve a vital physiologic function in helping regulate low-density lipoprotein metabolism in humans (Alving and Wassef, 1999).

REFERENCES

Alving, C. R., and Wassef, N. M. (1999). Naturally occurring antibodies to cholesterol: A new theory of LDL cholesterol metabolism. *Immunol. Today* 20, 362–366.

Asa, P. B., Cao, Y., and Garry, R. F. (2000). Antibodies to squalene in Gulf War Syndrome. *Exp. Mol. Pathol.* 68, 55–64, doi:10.1006/exmp.1999.2295.

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GULF WAR and HEALTH

VOLUME 1

Depleted Uranium, Pyridostigmine Bromide, Sarin, Vaccines

Carolyn E. Fulco, Catharyn T. Liverman, Harold C. Sox, *Editors*

Committee on Health Effects Associated with Exposures During the Gulf War

Division of Health Promotion and Disease Prevention

INSTITUTE OF MEDICINE

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Contents

TAB 2

CONTENTS

SQUALENE

The committee was asked to examine the literature on potential health effects of squalene since it has been raised as an issue of concern to Gulf War veterans. A recent GAO (1999a) report found that at the time of the Gulf War, DoD had concerns about having a sufficient quantity of the anthrax vaccine and sufficient time to fully immunize the troops (GAO, 1999a). However, DoD has stated that it decided against the use of novel adjuvant formulations (e.g., formulations with squalene) because of lengthy FDA relicensure requirements (GAO, 1999a). The following section provides a brief overview of the animal and human studies that have been conducted and concludes with the committee's thoughts on directions for future research. The committee was not asked to draw conclusions on the strength of the evidence for an association between exposure to squalene and adverse health effects.

Squalene[14] is a polyunsaturated terpene hydrocarbon that is widely distributed in nature. It is found in human sebum (a skin surface lipid) and is a precursor in the synthesis of human cholesterol (Final Report, 1982; Kelly, 1999). Its name stems from its abundance in shark (*Squalus* spp.) liver oil,[15] from which it was first isolated (Liu et al., 1976). Squalene also is found in other fish oils, olive oil (0.7 percent), wheat germ oil, rice bran oil, and many other foods.

For more than 25 years, squalene has been used commercially as an emollient for topical application of more than 300 cosmetic formulations, including suntan preparations, bath oil, eye makeup, hair preparations, lipstick, baby powder, and skin care preparations. Cosmetic concentrations range from less than 0.1 to more than 50 percent. Squalene also is available as a dietary supplement; as a constituent of certain pharmaceuticals, including suppositories; and as a carrier of lipid-soluble drugs (Final Report, 1982; Kelly, 1999). As described below, squalene is being investigated for a number of potential medical purposes.

Dietary Intake, Absorption, Distribution, and Metabolism

In the 1970s, the average dietary intake of squalene in the United States was calculated at 24 mg per day (given a daily dietary intake of 2,000 calories) (Liu et al., 1976). Olive oil is particularly rich in squalene. Among Asians, shark liver oil supplements containing squalene are popular over-the-counter folk remedies (Asnis et al., 1993). The average total squalene exposure of humans from all routes of administration does not appear to have been studied. In case studies, excessive ingestion of squalene from dietary supplements has led to lipoid pneumonia (Asnis et al., 1993).

[14]Its chemical formula is 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene.

[15]Squalene concentrations in shark liver oil range between 50 and 80 percent (Liu et al., 1976).

Squalene is absorbed through several routes of administration, depending on the species (Final Report, 1982). In mice, squalene penetrates slowly and poorly through the skin at a rate of 0.12 nmol/cm^2 per minute (Final Report, 1982). Subcutaneous administration in rabbits leads to increases of stored squalene in liver, muscle, and skin (Final Report, 1982). Virtually all squalene administered orally to rats (96–100 percent) is unabsorbed (Albro and Thomas, 1970).

In humans, about 60 percent of dietary squalene is absorbed through the gastrointestinal tract, with the remainder excreted in feces (Strandberg et al., 1990). A significant fraction of absorbed squalene is converted into cholesterol. Squalene is distributed throughout human tissues, with greatest concentrations in skin and fat (Kelly, 1999). Squalene in human serum comes from endogenous cholesterol synthesis and from diet (Strandberg et al., 1990; Kelly, 1999). Peak serum levels are attained 9–12 hours after ingestion (Gylling and Miettinen, 1994).

Animal Studies

There are few published studies of squalene toxicity in animals or humans (Kelly, 1999). Kamimura and colleagues (1989) examined subacute toxicity in dogs after a single oral squalene dose of 1,200 mg/kg. Over the next 3 months, accumulation was noted in several tissues, especially liver, but there were no signs of toxicity based on testing of serum and liver function. In contrast to humans, who absorb 60 percent of ingested squalene, this study reported that dogs absorb a relatively small percentage and excrete most in feces (83 percent). Thus, the relevance of this study to humans is unclear.

Squalene's toxicity is considered low, with an oral LD_{50} (median lethal dose) in mice at greater than 50 ml/kg (Final Report, 1982). No toxic responses were noted after subcutaneous and intramuscular injections of 0.5 ml per 20g mouse (25 ml/kg) of squalane ($C_{30}H_{62}$), a saturated and more stable version of squalene.

In a neuropathology study, squalene was administered subcutaneously to 10 rats (and 5 control rats) at a dose of 20 g/kg body weight for 4 consecutive days (Gajkowska et al., 1999). The rats' peripheral and central nervous systems were examined via electron microscopy 7 or 30 days from initiation of the experiment. After 7 days, disturbances in the myelin sheath were observed; these disturbances were more pronounced at 30 days. There was some swelling of Schwann cells in the peripheral nervous system. Fibroblasts were activated and showed signs of increased collagen production. In the central nervous system, squalene triggered swelling of astrocytes in white matter and in the hippocampus, especially near blood vessels. Lipid droplets accumulated in myelin in both the central and the peripheral nervous systems.

The pertinence of this neuropathology study is difficult to gauge because the dose was extremely high and the report provided minimal information about the study's methodology (especially handling of controls). Additionally, it is not uncommon to detect occasional astrocytic or neuronal swelling or mitochondrial swelling in electron micrographs of normal tissue. Further, damage appeared to be localized, not global, targeting, for example, a single myelin fiber or axon.

Squalene has attracted the interest of arthritis researchers because of its ability to activate the immune system nonspecifically. It was one of the constituents used in the 1970s to create the first animal models of multiple sclerosis, known as experimental allergic encephalomyelitis (EAE) (Beck et al., 1976). Squalene is one of several adjuvants (such as incomplete Freund's adjuvant) found to induce arthritis in *susceptible* rat strains and has been used in the generation of animal models of arthritis (Whitehouse et al., 1974; Lorentzen, 1999). The effect is so pronounced that researchers have coined the term "squalene-induced arthritis." After a single intraarticular injection of 50 μL squalene into Lewis (Yoshino, 1996) and Dark Agouti rats (Yoshino and Yoshino, 1994), animals experienced moderate joint inflammation by day 6, followed by more severe chronic arthritis by day 21. The inflammation was marked by joint swelling and infiltration of $CD5^+$ and $\alpha\beta^+$ T cells. Similarly, intradermal injection of 200 μL squalene into Dark Agouti rats produced arthritis (Lorentzen, 1999). Although the mechanisms are not fully understood, the inflammation is blocked by agents that suppress T cells (Yoshino, 1996; Sverdrup et al., 1998). Animal studies do not report whether injection of squalene produces antisqualene antibodies.

In summary, there is limited published information about squalene toxicity. The human relevance of what has been published is unclear because of species differences in absorption. Squalene has been found to produce arthritis and neuropathology under select conditions in animals; the relevance to humans of these toxicity findings is uncertain.

Use of Squalene as a Vaccine Adjuvant

Squalene is currently being studied for a number of medical purposes including treatment of hypercholesterolemia (Chan et al., 1996); as an antidote to reduce the toxicity of accidentally ingested drugs (Kelly, 1999); and as an adjunctive therapy in cancer treatment to potentiate the cytotoxic activity of some chemotherapeutic agents (Kelly, 1999). The area of research that is of particular relevance to this chapter is the use of squalene as a vaccine adjuvant or as a component of a vaccine adjuvant.

The dose of an adjuvant is typically small (in the microgram range), and the route of administration is usually intramuscular. Squalene has been tested primarily as one component of the vaccine adjuvant MF59. MF59 is an oil-in-water microemulsion, consisting of squalene, polysorbate 80 (Tween 80, polyoxethylene sorbitan monooleate), and sorbitan trioleate (Graham et al., 1996). FDA has not yet approved any experimental vaccines with squalene-containing adjuvants.

The safety and efficacy of MF59 has been tested in a number of animal species with recombinant and natural antigens. Both short-term (approximately 2 weeks) and long-term (8 months) studies have been conducted and have detected some minor and transient changes in clinical laboratory parameters and histopathology (Ott et al., 1995). As described earlier in this chapter, a study by Ivins and colleagues (1995) on numerous combinations of adjuvants with the

purified anthrax protective antigen found adverse effects from one of the adjuvant combinations containing squalene; other adjuvants containing squalene did not elicit adverse reactions. Tests of an HIV candidate vaccine with MF59 found no embryotoxic or teratogenic effects in dogs or rabbits (Ott et al., 1995).

Clinical studies of MF59 and other squalene-containing adjuvants have been conducted with candidate malaria, HSV (herpes simplex virus), HIV (human immunodeficiency virus), and influenza vaccines (Ott et al., 1995; GAO, 1999a). Study populations for the clinical trials have included adults, elderly, and children and infants (Ott et al., 1995).

HIV Vaccine Trials

Keefer and colleagues (1996) investigated the safety and immunogenicity of a candidate HIV-1 vaccine in combination with MF59, with or without an additional immune modulator, MTP–PE (muramyl tripeptide linked covalently with dipalmitoyl phosphatidylethanolamine). Vaccination with the candidate vaccine Env 2-3 in MTP–PE/MF59 was associated with significant adverse effects; severe, though short-lived, systemic and/or local reactions occurred in 15 of 30 vaccinees. In contrast, Env 2-3 in MF59 without MTP–PE was relatively well tolerated; severe local and/or systemic reactions occurred in only 2 of 18 subjects. There were no severe reactions in the eight subjects that received MF59 alone.

Graham and colleagues (1996) evaluated the safety and immunogenicity of another candidate vaccine for HIV, the recombinant glycoprotein 120, formulated with MF59 with or without MTP–PE. Vaccines that contained MTP–PE caused a greater number of moderate or severe local and systemic reactions (of 16 participants, 4 had local reactions and 13 had systemic reactions) than did vaccine formulated with MF59 alone. Of 16 vaccinees, 7 had local reactions and 0 had systemic reactions.

The National Institute of Allergy and Infectious Diseases (NIAID)-sponsored AIDS Vaccine Evaluation Group examined safety data from 1,398 HIV-negative, healthy volunteers who were enrolled in 25 multicenter, randomized double-blind studies evaluating 11 HIV candidate vaccines (Keefer et al., 1996). The study examined the adverse effects of a number of adjuvants, including MF59 and MF59 formulated with the biological response modifier MTP–PE. MTP–PE was associated with moderate to severe local reactions as well as with self-limited severe systemic reactions that resolved within 2–3 days. The same vaccines in the MF59 emulsion alone were well tolerated (Keefer et al., 1996).

Influenza Vaccine Trials

The safety and efficacy of MF59 have been evaluated in pilot studies (Keitel et al., 1993) and clinical trials (Martin, 1997; Menegon et al., 1999; Minutello et al., 1999). A study by Martin (1997) assembled data from eight randomized controlled clinical trials over four influenza seasons; 984 elderly

volunteers (older than 65 years) received the adjuvanted vaccine, and 823 elderly volunteers received a conventional influenza vaccine. More than 20 percent of the volunteers who received the adjuvanted vaccine had local reactions. Myalgia was the only systemic effect to have been significantly more common in those receiving the vaccine with a squalene-containing adjuvant (3.9 percent) than those receiving the vaccine without the adjuvant (1.8 percent). All adverse events were recorded for 1 week after vaccination. Hospitalization and mortality were followed during the influenza season. The group receiving the adjuvanted vaccine had similar hospitalization rates and lower mortality than subjects receiving the conventional vaccine.

To date, clinical studies of the MF59 adjuvant that contains squalene have not shown any adverse health effects beyond transient acute effects.

Gulf War Issues

A recent study by Asa and colleagues (2000) reports on the development of an anti-squalene antibody assay to detect antibodies to squalene in the circulation. Blood samples from 144 Gulf War era veterans or military employees, 48 blood donors, 40 patients with systemic lupus erythematosus (SLE), 34 patients with silicone breast implants, and 30 patients with chronic fatigue syndrome (CFS) were studied for squalene antibodies. The study reports that a blinded test of serum samples found antibodies to squalene in more than 95 percent of 38 veterans deployed to the Gulf War who developed chronic illness symptoms; in all of the 6 veterans not deployed to the Gulf War who developed chronic illness symptoms; and in none of 12 veterans deployed to the Gulf War who were healthy. In an unblinded test, the study reported antibody reactivity to squalene in 5 percent of blood donors, 10 percent of patients with SLE, 10 percent of patients with silicone breast implants, and 15 percent of patients with CFS.

This study has several shortcomings. The subjects were self-selected, rather than being chosen at random from a larger sample, which can introduce substantial selection bias and does not allow inferences to the broader population of Gulf War veterans. Sample sizes were small, and the study may suffer from misclassification errors since the group of Gulf War veterans categorized as healthy (n = 12) was not devoid of individuals with serious symptoms (1 had fibromyalgia, 1 had thyroid disease, 3 had memory loss, and 4 had chronic fatigue). Further, the report provides inadequate evidence that the assay is able to accurately detect antibodies to squalene. Many of the methods used in the study are not described; as a result it is not possible to fully assess the study's methodology or to reproduce the assay. The study did not attempt to demonstrate that the substance giving the positive response in the assay was found in the immunoglobulin G (IgG) fraction of serum where antibodies are found. Further, the authors did not show that the assay was specific to squalene. To prove the specificity of the assay, the investigators would have had to show inhibition, in a dose–response manner, with squalene and no inhibition with other substances, as is seen in most reports of new enzyme-linked immunosorbent assays (ELISAs).

The committee does not regard this study as providing evidence that the investigators have successfully measured antibodies to squalene.

Future Research Directions Regarding Squalene

As squalene continues to be investigated for a number of clinical uses, ongoing toxicity studies will provide the additional information that is needed about its toxicity, both in animals and in humans. It will be important to examine the relevance of animal studies because of species differences in the absorption of squalene and the susceptibility of certain strains of animals to squalene's effects. In considering future research directions, the committee focused on squalene's potential use as a vaccine adjuvant. Research questions that remain to be addressed include the following:

- What types of immune responses does exogenous squalene evoke?
- Does the immune response differ with the route of administration or entry (i.e., oral, cutaneous, intramuscular)?
- How does the response vary according to the dose of squalene?
- Is the presence of antibodies to squalene abnormal, and if so, what is their functional significance?
- Could antibodies to squalene represent the consequences of, rather than the cause of, a pathological process?

CONCLUSIONS

The committee felt it would be helpful to the reader to restate the conclusions from this chapter. The conclusions listed below are identical to those made at the end of the respective sections of this chapter.

Anthrax Vaccine

There is a paucity of published peer-reviewed literature on the safety of the anthrax vaccine. The committee located only one randomized peer-reviewed study of the type of anthrax vaccine used in the United States (Brachman et al., 1962). However, the formulation of the vaccine used in that study differs somewhat from the vaccine given to Gulf War veterans (and currently in use). The Brachman study (and other early experimental studies) found transient local and systemic effects (primarily erythema, edema, induration) of the anthrax vaccine. There was no long-term monitoring for adverse outcomes. The committee did not compare the incidence of transient effects with other vaccines.

Studies of the anthrax vaccine have not used active surveillance to systematically evaluate long-term health outcomes. This situation is unfortunately typi-

cal for all but a few vaccines. The committee strongly encourages active monitoring to evaluate the long-term safety of the anthrax vaccine.

To date, published studies have reported no significant adverse effects of the vaccine, but the literature is limited to a few short-term studies. Reviewing the large body of results that have not yet been published may enable more definitive conclusions about the vaccine's safety. The committee strongly urges the investigators conducting studies on the safety of the anthrax vaccine to submit their results to peer-reviewed scientific journals for publication.

The committee's findings are best regarded as an early step in the complex process of understanding the vaccine's safety, which began with the vaccine's licensure in 1970 and the 1985 FDA advisory panel finding that categorized the anthrax vaccine as safe and effective. Active long-term monitoring of large populations will provide further information for documenting the relative safety of the anthrax vaccine.

The committee concludes that there is sufficient evidence of an association between anthrax vaccination and transient acute local and systemic effects (e.g., redness, swelling, fever) typically associated with vaccination.

The committee concludes that there is inadequate/insufficient evidence to determine whether an association does or does not exist between anthrax vaccination and long-term adverse health effects.

The latter finding means that the evidence reviewed by the committee is of insufficient quality, consistency, or statistical power to permit a conclusion regarding the presence or absence of an association between the vaccine and a health outcome in humans.

Botulinum Toxoid

Studies have noted transient local and systemic effects of the botulinum toxoid vaccine. However, studies of the botulinum toxoid vaccine have not used active surveillance to systematically evaluate long-term health outcomes. This situation is unfortunately typical for all but a few vaccines.

The committee concludes that there is sufficient evidence of an association between botulinum toxoid vaccination and transient acute local and systemic effects (e.g., redness, swelling, fever) typically associated with vaccination.

The committee concludes that there is inadequate/insufficient evidence to determine whether an association does or does not exist between botulinum toxoid vaccination and long-term adverse health effects.

The latter finding means that the evidence reviewed by the committee is of insufficient quality, consistency, or statistical power to permit a conclusion regarding the presence or absence of an association between the vaccine and a health outcome in humans.

Multiple Vaccinations

Certain multiple vaccination regimens can lead to suboptimal antibody responses, but there is little evidence, largely because of a lack of active monitoring, of other adverse clinical or laboratory consequences beyond the transient local and systemic effects seen frequently with any vaccination.

No long-term identifiable clinical sequelae attributable to intense long-term immunization occurred in the Fort Detrick cohort. There was some evidence of a chronic inflammatory response, but these changes cannot necessarily be attributed to the vaccinations, since the workers studied were occupationally exposed to a number of virulent microbes. This series of longitudinal clinical studies also had several shortcomings. However, the studies are valuable because careful monitoring did not disclose any evidence of serious unexplained illness in a cohort that received a series of intense vaccination protocols over many years.

The U.K. Gulf War studies provide some limited evidence of an association between multiple vaccinations and long-term multisymptom outcomes, particularly for vaccinations given during deployment (Unwin et al., 1999; Hotopf et al., 2000). There are some limitations and confounding factors in these studies, and further research is needed.

The committee concludes that there is inadequate/insufficient evidence to determine whether an association does or does not exist between multiple vaccinations and long-term adverse health effects.

This finding means that the evidence reviewed by the committee is of insufficient quality, consistency, or statistical power to permit a conclusion regarding the presence or absence of an association between multiple vaccinations and health outcomes in humans.

REFERENCES

Ahmed SA, Hissong BD, Verthelyi D, Donner K, Becker K, Karpuzoglu-Sahin E. 1999. Gender and risk of autoimmune diseases: Possible role of estrogenic compounds. *Environ Health Perspect* 107(Suppl 5):681–686.

Aho K, Wager O. 1961. Production of anti-antibodies in rabbits. *Ann Med Exper Fenn* 39:79–87.

Aho K, Konttinen A, Wager O. 1962. Transient appearance of the rheumatoid factor in connection with prophylactic vaccinations. *Acta Pathologica et Microbiologica Scandinavica* 56(4):478–479.

Aho K, Somer T, Salo OP. 1967. Rheumatoid factor and immuno-conglutinin responses following various vaccinations. *Proc Soc Exp Biol Med* 124(1):229–233.

Albert LJ, Inman RD. 1999. Molecular mimicry and autoimmunity. *N Engl J Med* 341:2068–2074.

Albro PW, Thomas R. 1970. Absorption of aliphatic hydrocarbons by rats. *Biochem Biophys Acta* 291:437–446.

Anderson JH Jr, Lewis GE Jr. 1981. Clinical evaluation of botulinum toxoids. In: Lewis GH Jr, ed. *Biomedical Aspects of Botulism*. New York: Academic Press.

Anderson RE. 1971. Disseminated amyloidosis in germfree mice. *Am J Pathol* 65:43–50.

Asa PB, Cao Y, Garry RF. 2000. Antibodies to squalene in Gulf War syndrome. *Exp Mol Pathol* 68(1):55–64.

Asnis DS, Saltzman HP, Melchert A. 1993. Shark oil pneumonia. An overlooked entity. *Chest* 103(3):976–977.

Azar HA. 1968. Significance of adjuvant-induced plasmacytomas of mice in relation to the pathogenesis of human multiple myeloma. *Ann Allergy* 26(6):293–298.

Barnard JP, Friedlander AM. 1999. Vaccination against anthrax with attenuated recombinant strains of *Bacillus anthracis* that produce protective antigen. *Infect Immunol* 67(2):562–567.

Barnes PJ. 1999. Therapeutic strategies for allergic diseases. *Nature* 402(6760 Suppl): B31–B38.

Bavari S, Pless DD, Torres ER, Lebeda FJ, Olson MA. 1998. Identifying the principal protective antigenic determinants of type A botulinum neurotoxin. *Vaccine* 16(19): 1850–1856.

Beck FW, Whitehouse MW, Pearson CM. 1976. Improvements for consistently inducing experimental allergic encephalomyelitis (EAE) in rats: I. Without using mycobacterium. II. Inoculating encephalitogen into the ear. *Proc Soc Exp Biol Med* 151(3): 615–622.

BioPort. 1999. *Anthrax Vaccine Adsorbed, Package Insert*. [Online]. Available: http://www.bioportcorp.com/Anthraxins.htm [accessed June 1999].

Borodko SL, Samsonovich LG. 1965. *Duration of Immunity in Guinea Pigs Inoculated with Combined Live Vaccine Against Plague, Tularemia, Brucellosis and Anthrax*. Available from the National Technical Information Service. AD-638 588/XAB.

Brachman PS, Gold H, Plotkin S, Fekety FR, Werrin M, Ingraham NR. 1962. Field evaluation of a human anthrax vaccine. *Am J Public Health* 52:632–645.

Brin MF. 1997. Botulinum toxin: Chemistry, pharmacology, toxicity, and immunology. *Muscle Nerve Suppl* 6:S146–S168.

Byrne MP, Smith TJ, Montgomery VA, Smith LA. 1998. Purification, potency, and efficacy of the botulinum neurotoxin type A binding domain from *Pichia pastoris* as a recombinant vaccine candidate. *Infect Immun* 66(10):4817–4822.

Cardella MA. 1964. *Botulinum Toxoids*. Frederick, MD. Available from the National Technical Information Service. AD-443 673/9/XAB.

Cardoso F, Jankovic J. 1995. Clinical use of botulinum neurotoxins. *Curr Top Microbiol Immunol* 195:123–141.

CDC (Centers for Disease Control and Prevention). 1994a. General recommendations on immunization. Recommendations of the Advisory Committee on Immunization Practices. *MMWR* 43(RR-1).

CDC (Centers for Disease Control and Prevention). 1994b. *Progress Report #28 BB-IND 161: Pentavalent Botulinum Phosphate Adsorbed (March 2, 1993 to March 1, 1994)*. Atlanta, GA.

CDC (Centers for Disease Control and Prevention). 1995. *Progress Report #29 BB-IND 161: Pentavalent Botulinum Phosphate Adsorbed (March 2, 1994 to March 1, 1995)*. Atlanta, GA.

CDC (Centers for Disease Control and Prevention). 1996. *Progress Report #30 BB-IND 161: Pentavalent Botulinum Phosphate Adsorbed (March 2, 1995 to March 1, 1996)*. Atlanta, GA.

CDC (Centers for Disease Control and Prevention). 1997. *Progress Report #31 BB-IND 161: Pentavalent Botulinum Phosphate Adsorbed (March 2, 1996 to March 1, 1997)*. Atlanta, GA.

CDC (Centers for Disease Control and Prevention). 1998. *Progress Report #32 BB-IND 161: Pentavalent Botulinum Phosphate Adsorbed (March 2, 1997 to March 1, 1998)*. Atlanta, GA.

CDC (Centers for Disease Control and Prevention). 1999a. Combination vaccines for childhood immunization. *MMWR* 48(RR05):1–15.

CDC (Centers for Disease Control and Prevention). 1999b. *Vaccine Adverse Event Reporting System (VAERS)*. [Online]. Available: http://www.cdc.gov/nip/vaers.htm [accessed June 1999].

CDC (Centers for Disease Control and Prevention). 2000. Surveillance for adverse effects associated with anthrax vaccination—U.S. Department of Defense, 1998–2000. *MMWR* 49(16):341–345.

Chan P, Tomlinson B, Lee CB, Lee YS. 1996. Effectiveness and safety of low-dose pravastatin and squalene, alone and in combination, in elderly patients with hypercholesterolemia. *J Clin Pharmacol* 36(5):422–427.

Chen RT, Haber P, Mullen JR. 1995. Surveillance of the safety of simultaneous administration of vaccines. *Ann NY Acad Sci* 754:309–320.

Choo S, Finn A. 1999. Pediatric combination vaccines. *Current Opin Pediatr* 11(1):14–20.

Christopher GW, Cieslak TJ, Pavlin JA, Eitzen EM Jr. 1997. Biological warfare. A historical perspective. *JAMA* 278(5):412–417.

Classen JB. 1996. The timing of immunization affects the development of diabetes in rodents. *Autoimmunity* 24(3):137–145.

Claypool GR. 1999. *The Anthrax Vaccine Immunization Program*. Statement at the July 21, 1999 hearing of the Subcommittee on National Security, Veterans Affairs, and International Relations, Committee on Government Reform, U.S. House of Representatives. U.S. Army Medical Corps, Deputy Assistant Secretary for Health Operations Policy. Washington, DC.

Clayton MA, Clayton JM, Brown DR, Middlebrook JL. 1995. Protective vaccination with a recombinant fragment of *Clostridium botulinum* neurotoxin serotype A expressed from a synthetic gene in *Escherichia coli*. *Infect Immun* 63(7):2738–2742.

Committee on Veterans' Affairs, U.S. Senate. 1998. *Report of the Special Investigation Unit on Gulf War Illnesses*. 105th Congress, 2nd session. Washington, DC: Government Printing Office. S.PRT 105-39.

Coombs RR, Gell PG. 1968. Classification of allergic reactions responsible for clinical hypersensitivity and disease. In: Gell PG, Coombs RR, eds. *Clinical Aspects of Immunology*. 2nd edition. Oxford: Blackwell.

Cooper GS, Miller FW, Pandey JP. 1999. The role of genetic factors in autoimmune disease: Implications for environmental research. *Environ Health Perspect* 107(Suppl 5):693–700

Darlow HM, Belton FC, Camb BA. 1956. The use of anthrax antigen to immunise man and monkey. *Lancet* 2:476–479.

Davidson I. 1976. An international survey of clostridial sera and vaccines. *Dev Biol Stand* 32:3–14.

DeAmmon IA Jr, Klein F, Lincoln RE, Mahlandt BG, Fernelius AL. 1961. Immunological studies of anthrax. I. An index to determine quantitative immunization. *J Immunol* 87:233–239.

Demicheli V, Rivetti D, Deeks JJ, Jefferson T, Pratt M. 1998. The effectiveness and safety of vaccines against human anthrax: A systematic review. *Vaccine* 16(9–10): 880–884.

Dohms JE, Allen PH, Cloud SS. 1982. The immunization of broiler chickens against type C botulism. *Avian Dis* 26(2):340–345.

Ellenberg SS. 1999. *Statement at the July 21, 1999 Hearing of the Subcommittee on National Security, Veterans Affairs, and International Relations, Committee on Government Reform, U.S. House of Representatives.* Rockville, MD: Food and Drug Administration.

Ellis RW. 1996. Challenges in the development of combination vaccines. In: Cohen S, Shafferman A, eds. *Novel Strategies in Design and Production of Vaccines.* New York: Plenum Press. Pp. 127–132.

Ezzell JWJ, Abshire TG. 1988. Immunological analysis of cell-associated antigens of *Bacillus anthracis*. *Infect Immun* 56(2):349–356.

Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL, Longo DL, eds. 1998. *Harrison's Principles of Internal Medicine.* 14th edition. New York: McGraw Hill.

FDA (Food and Drug Administration). 1985. Biological products: Bacterial vaccines and toxoids: Implementation of efficacy review. Proposed Rule. *Federal Register* 50(240):51002–51117.

Final Report. 1982. Final report on the safety assessment of squalane and squalene. *J Am Coll Toxicol* 1(2):37–56.

Fiock MA, Devine LF, Gearinger NF, Duff JT, Wright GG, Kadull PJ. 1962. Studies on immunity to toxins of *Clostridium botulinum*. VIII. Immunological response of man to purified bivalent AB botulinum toxoid. *J Immunol* 88:277–283.

Fiock MA, Cardella MA, Gearinger NF. 1963. Studies on immunity to toxins of *Clostridium botulinum*. X. Immunologic response of man to purified pentavalent ABCDE botulinum toxoid. *J Immunol* 90:697–702.

Franz DR, Jahrling PB, Friedlander AM, McClain DJ, Hoover DL, Bryne WR, Pavlin JA, Christopher GW, Eitzen EM Jr. 1997. Clinical recognition and management of patients exposed to biological warfare agents. *JAMA* 278(5):399–411.

Friedlander AM. 1986. Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. *J Biol Chem* 261(16):7123–7126.

Friedlander AM. 1997. Anthrax. In: Zajtchuk R, Bellamy R, eds. *Textbook of Military Medicine: Medical Aspects of Chemical and Biological Warfare.* Washington, DC: Office of the Surgeon General, Department of the Army. Pp. 467–478.

Friedlander AM, Pittman PR, Parker GW. 1999. Anthrax vaccine: Evidence for safety and efficacy against inhalational anthrax. *JAMA* 282(22):2104–2106.

Fukuda K, Nisenbaum R, Stewart G, Thompson WW, Robin L, Washko RM, Noah DL, Barrett DH, Randall B, Herwaldt BL, Mawle AC, Reeves WC. 1998. Chronic multisymptom illness affecting Air Force veterans of the Gulf War. *JAMA* 280(11):981–988.

Gajkowska B, Smialek M, Ostrowski RP, Piotrowski P, Frontczak-Baniewicz M. 1999. The experimental squalene encephaloneuropathy in the rat. *Exp Toxicol Pathol* 51(1):75–80.

GAO (U.S. General Accounting Office). 1999a. *Gulf War Illnesses: Questions About the Presence of Squalene Antibodies in Veterans Can Be Resolved.* GAO/T-NSIAD-99-5. Washington, DC: GAO.

GAO (U.S. General Accounting Office). 1999b. *Medical Readiness: Issues Concerning the Anthrax Vaccine.* Statement of Kwai-Cheung Chan, Director, Special Studies and Evaluations, National Security and International Affairs Division, before the Subcommittee on National Security, Veterans' Affairs, and International Relations, Committee on Government Reform, House of Representatives. GAO/T-NSIAD-99-226. Washington, DC: GAO.

GAO (U.S. General Accounting Office). 1999c. *Medical Readiness: Safety and Efficacy of the Anthrax Vaccine.* Statement of Kwai-Cheung Chan, Director, Special Studies and Evaluations, National Security and International Affairs Division, before the Subcommittee on National Security, Veterans' Affairs, and International Relations, Committee on Government Reform, House of Representatives. GAO/T-NSIAD-99-148. Washington, DC: GAO.

GAO (U.S. General Accounting Office). 1999d. *Anthrax Vaccine: Safety and Efficacy Issues.* GAO/NSAID-00-48. Washington, DC: GAO.

Gendon YZ. 1958. *Changes in the Fractional Composition of the Serum Proteins of Mice and Guinea-Pigs on Immunization with Type A Clostridium Botulinum Toxoids.* Available from the National Technical Information Service. AD-682 631/XAB.

Germuth FG. 1953. A comparative histologic and immunologic study in rabbits of induced hypersensitivity of the serum sickness type. *J Exp Med* 97:257–282.

Giles RB Jr, Calkins E. 1958. The relationship of serum hexosamine, globulins, and antibodies to experimental amyloidosis. *J Clin Invest* 37:846–857.

Goldenthal KL, Burns DL, McVittie LD, Lewis BP, Williams JC. 1995. Overview: Combined vaccines and simultaneous administration. Past, present, and future. *Ann NY Acad Sci* 754:xi–xv.

Gorski J, Motz J. 1984. Safety and immunogenic value of the vaccines against botulism and distemper simultaneously administered to the mink. *Bull Vet Inst Pulawy* 27(1–4):16–22.

Grabenstein JD. 1990. Drug interactions involving immunologic agents. Part 1. Vaccine-vaccine, vaccine-immunoglobulin, and vaccine-drug interactions. *DICP* 24(1):67–81.

Graham BS, Keefer MC, McElrath MJ, Gorse GJ, Schwartz DH, Weinhold K, Matthews TJ, Esterlitz JR, Sinangil F, Fast PE, NIAID AIDS Vaccine Evaluation Group. 1996. Safety and immunogenicity of a candidate HIV-1 vaccine in healthy adults: Recombinant glycoprotein (rgp) 120. A randomized, double-blind trial. *Ann Intern Med* 125(4):270–279.

Gu M-L, Leppla SH, Klinman DM. 1999. Protection against anthrax toxin by vaccination with a DNA plasmid encoding anthrax protective antigen. *Vaccine* 17(4):340–344.

Gulrajani TS, Misra RP, Verma JC, Ahuja ML. 1968. Effect of adjuvants on immunising efficacy of *Bacillus anthracis* protective antigen. *Indian Vet J* 45(6):465–476.

Gusman BS, Migulina TV. 1967. *Morphology of Immunogenesis During Experimental Anthrax Vaccination.* Available from the National Technical Information Service. AD-675 373/XAB.

Gylling H, Miettinen TA. 1994. Postabsorptive metabolism of dietary squalene. *Atherosclerosis* 106(2):169–178.

Hanna PC, Acosta D, Collier RJ. 1993. On the role of macrophages in anthrax. *Proc Natl Acad Sci USA* 90:10198–10201.

Holt PG, Macaubas C, Stumbles PA, Sly PD. 1999. The role of allergy in the development of asthma. *Nature* 402(6760 Suppl):B12–B17.

Hotopf M, David A, Hull L, Ismail K, Unwin C, Wessely S. 2000. Role of vaccinations as risk factors for ill health in veterans of the Gulf war: Cross sectional study. *BMJ* 320:1363–1367.

Ibrahim KH, Brown G, Wright DH, Rotschafer JC. 1999. *Bacillus anthracis*: Medical issues of biologic warfare. *Pharmacotherapy* 19(6):690–701.

Insel RA. 1995. Potential alterations in immunogenicity by combining or simultaneously administering vaccine components. *Ann NY Acad Sci* 754:35–47.

IOM (Institute of Medicine). 1991. *Adverse Effects of Pertussis and Rubella Vaccines*. Washington, DC: National Academy Press.

IOM (Institute of Medicine). 1994. *Adverse Events Associated with Childhood Vaccines: Evidence Bearing on Causality*. Washington, DC: National Academy Press.

IOM (Institute of Medicine). 1996. *Interactions of Drugs, Biologics, and Chemicals in U.S. Military Forces*. Washington, DC: National Academy Press.

IOM (Institute of Medicine). 1997. *Vaccine Safety Forum: Summaries of Two Workshops*. Washington, DC: National Academy Press.

IOM (Institute of Medicine). 2000. *Safety of the Anthrax Vaccine: A Letter Report*. Washington, DC: National Academy Press.

Ivins BE. 1988. *The Search for a New-Generation Human Anthrax Vaccine*. Ft. Detrick, MD: U.S. Army Medical Research Institute of Infectious Diseases. Available from the National Technical Information Service. AD-A190178.

Ivins BE, Ezzell JW Jr, Jemski J, Hedlund KW, Ristroph JD, Leppla SH. 1986. Immunization studies with attenuated strains of *Bacillus anthracis*. *Infect Immun* 52(2):454–458.

Ivins BE, Welkos SL, Little SF, Knudson GB. 1989. *Cloned Protective Activity and Progress in Development of Improved Anthrax Vaccines*. Ft. Detrick, MD: U.S. Army Medical Research Institute of Infectious Diseases. Available from the National Technical Information Service. AD-A216 312/9.

Ivins BE, Welkos SL, Knudson GB, Little SF. 1990. Immunization against anthrax with aromatic compound-dependent (Aro-) mutants of *Bacillus anthracis* and with recombinant strains of *Bacillus subtilis* that produce anthrax protective antigen. *Infect Immun* 58(2):303–308.

Ivins BE, Welkos SL, Little SF, Crummine MH, Nelson GO. 1992. Immunization against anthrax with *Bacillus anthracis* protective antigen combined with adjuvants. *Infect Immun* 60(2):662–668.

Ivins BE, Fellows PF, Pitt ML, Welkos SL. 1993. Experimental anthrax vaccines: Efficacy studies in guinea pigs. *Abstracts of the General Meeting of the American Society for Microbiology* 93(0):160.

Ivins BE, Fellows PF, Nelson GO. 1994. Efficacy of a standard human anthrax vaccine against *Bacillus anthracis* spore challenge in guinea-pigs. *Vaccine* 12(10):872–874.

Ivins B, Fellows P, Pitt L, Estep J, Farchaus J, Friedlander A, Gibbs P. 1995. Experimental anthrax vaccines: Efficacy of adjuvants combined with protective antigen against an aerosol *Bacillus anthracis* spore challenge in guinea pigs. *Vaccine* 13(18): 1779–1784.

Ivins BE, Pitt ML, Fellows PF, Farchaus JW, Benner GE, Waag DM, Little SF, Anderson GW Jr, Gibbs PH, Friedlander AM. 1998. Comparative efficacy of experimental anthrax vaccine candidates against inhalation anthrax in rhesus macaques. *Vaccine* 16(11–12):1141–1148.

Jaiswal TN, Mittal KR. 1979. Potency testing of anthrax spore vaccine (living) in guinea pigs. *Indian Vet J* 56(3):199–201.

Kamimura H, Koga N, Oguri K, Yoshimura H, Inoue H, Sato K, Ohkubo M. 1989. [Studies on distribution, excretion and subacute toxicity of squalene in dogs]. *Fukuoka Igaku Zasshi* 80:269–280.

Kaufmann AF, Fox MD, Kolb RC. 1973. Anthrax in Louisiana, 1971: An evaluation of the Sterne strain anthrax vaccine. *J Am Vet Med Assoc* 163(5):442–445.

Keefer MC, Graham BS, McElrath MJ, Matthews TJ, Stablein DM, Corey L, Wright PF, Lawrence D, Fast PE, Weinhold K, Hsieh RH, Chernoff D, Dekker C, Dolin R. 1996. Safety and immunogenicity of Env 2-3, a human immunodeficiency virus type 1 candidate vaccine, in combination with a novel adjuvant, MTP–PE/MF59. *AIDS Res Hum Retroviruses* 12(8):683–693.

Keitel W, Couch R, Bond N, Adair S, Van Nest G, Dekker C. 1993. Pilot evaluation of influenza virus vaccine (IVV) combined with adjuvant. *Vaccine* 11(9):909–913.

Kelly GS. 1999. Squalene and its potential clinical uses. *Alternative Med Rev* 4(1):29–36.

Keusch GT, Bart KJ. 1998. Immunization principles and vaccine use. In: Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL, Longo DL, eds. *Harrison's Principles of Internal Medicine*. 14th edition. New York: McGraw Hill.

King GE, Hadler SC. 1994. Simultaneous administration of childhood vaccines: An important public health policy that is safe and efficacious. *Pediatr Infect Dis J* 13:394–407.

Kiyatkin N, Maksymowych AB, Simpson LL. 1997. Induction of an immune response by oral administration of recombinant botulinum toxin. *Infect Immun* 65(11):4586–4591.

Klein F, Mahlandt BG, Lincoln RE, DeArmon IA Jr, Fernelius AL. 1961. Immunization as a factor affecting the course of septicemic anthrax. *Science* 133:1021–1022.

Klein F, DeArmon IA Jr, Lincoln RE, Mahlandt BG, Fernelius AL. 1962. Immunological studies of anthrax. II. Levels of immunity against *Bacillus anthracis* obtained with protective antigen and live vaccine. *J Immunol* 88:15–19.

Kolesov SG, Gutiman AA. 1968. *The Study of the Complications Resulting from the Application of STI Vaccine*. Available from the National Technical Information Service. AD-674 877/XAB.

Kolesov SG, Mikhailov NA, Borisovich YF. 1968. *Aluminum Hydroxide Vaccine Against Anthrax*. Translation of *Veterinariya (USSR)* 34(10):39–45 1957. Available from the National Technical Information Service. AD-672 433/XAB.

Kolksov SG, Mikhailov NA. 1959. *Studies of the Immunogenic Properties of the Aluminum Hydroxide Vaccine Against Anthrax and Testing It At-Large in the Practice*. Available from the National Technical Information Service. AD-640 528/XAB.

Kolosov SG, Borisovich YF. 1968. *Obtainance of Anthrax Strains for the Purpose of Prophylactic Vaccination*. Available from the National Technical Information Service. AD-674 926/XAB.

Kondo H, Kondo S, Murata R, Sakaguchi G. 1969. Antigenicity of *Clostridium botulinum* type-E formol toxoid. *Jpn J Med Sci Biol* 22(2):75–85.

Kurazono H, Shimozawa K, Sakaguchi G, Takahashi M, Shimizu T, Kondo H. 1985. Botulism among penned pheasants and protection by vaccination with C1 toxoid. *Res Vet Sci* 38(1):104–108.

Leppla SH. 1982. Anthrax toxin edema factor: A bacterial adenylate cyclase that increases cyclic AMP concentrations in eukaryotic cells. *Proc Natl Acad Sci USA* 79: 3162–3166.

Leppla S, Friedlander AM, Singh EM, Bhatnagar R. 1990. A model for anthrax toxic action at the cellular level. *Salisbury Medical Bulletin* 68(Special Supplement):41–43.

Lewis GE Jr, ed. 1981. *Biomedical Aspects of Botulism*. New York: Academic Press.

Lindley WH. 1963. Anthrax vaccination. *J Am Vet Med Assoc* 142(6):621–623.

Little SF, Knudson GB. 1986. Comparative efficacy of *Bacillus anthracis* live spore vaccine and protective antigen vaccine against anthrax in the guinea pig. *Infect Immun* 52(2):509–512.

Liu GC, Ahrens EH Jr, Schreibman PH, Crouse JR. 1976. Measurement of squalene in human tissues and plasma: Validation and application. *J Lipid Res* 17(1):38–45.

Lopez GP. 1998. Botulinum toxin. In: Wexler P, ed. *Encyclopedia of Toxicology*. San Diego: Academic Press. Pp. 184–185.

Lorentzen JC. 1999. Identification of arthritogenic adjuvants of self and foreign origin. *Scand J Immunol* 49(1):45–50.

Martin JT. 1997. Development of an adjuvant to enhance the immune response to influenza vaccine in the elderly. *Biologicals* 25(2):209–213.

Mathews AG. 1976. Antitoxin responses to *Clostridium botulinum* vaccines types C and D in guinea pigs. *Dev Biol Stand* 32:193–201.

McBride BW, Mogg A, Telfer JL, Lever MS, Miller J, Turnbull PC, Baillie L. 1998. Protective efficacy of a recombinant protective antigen against *Bacillus anthracis* challenge and assessment of immunological markers. *Vaccine* 16(8):810–817.

Menegon T, Baldo V, Bonello C, Dalla Costa D, Di Tommaso A, Trivello R. 1999. Influenza vaccines: Antibody responses to split virus and MF59-adjuvanted subunit virus in an adult population. *Eur J Epidemiol* 15(6):573–576.

Middlebrook JL. 1995. Protection strategies against botulinum toxin. *Adv Exp Med Biol* 383:93–98.

Middlebrook JL, Brown JE. 1995. Immunodiagnosis and immunotherapy of tetanus and botulinum neurotoxins. *Curr Top Microbiol Immunol* 195:89–122.

Middlebrook JL, Lapenotiere H, Clayton M, Brown D. 1994. Development of a molecularly engineered vaccine for botulinum toxins. *FEMS Symposium* 73:531–532.

Mikhailova IM. 1966. *Cl. Botulinum F. Report II, Biochemical Properties. A Study of Toxin- and Toxoid-Formation*. Available from the National Technical Information Service. AD-652 659/XAB.

Miller FW. 1999. Genetics of environmentally associated rheumatic disease. In: Kaufman LD, Varga J, eds. *Rheumatic Diseases and the Environment*. New York: Oxford University Press. Pp. 33–41.

Miller J, McBride BW, Manchee RJ, Moore P, Baillie LW. 1998. Production and purification of recombinant protective antigen and protective efficacy against *Bacillus anthracis*. *Lett Appl Microbiol* 26(1):56–60.

Minutello M, Senatore F, Cecchinelli G, Bianchi M, Andreani T, Podda A, Crovari P. 1999. Safety and immunogenicity of an inactivated subunit influenza virus vaccine combined with MF59 adjuvant emulsion in elderly subjects, immunized for three consecutive influenza seasons. *Vaccine* 17(2):99–104.

Nass N. 1999. Anthrax vaccine: Model of a response to the biological warfare threat. *Infect Dis Clin North Am* 13(1):187–208.

OSAGWI (Office of the Special Assistant for Gulf War Illnesses). 1999. *Military Medical Recordkeeping During and After the Gulf War: Interim Report*. Washington, DC: U.S. Department of Defense.

Ott G, Barchfeld GL, Chernoff D, Radhakrishnan R, van Hoogevest P, Van Nest G. 1995. Design and evaluation of a safe and potent adjuvant for human vaccines. In: